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Genotoxic and mutagenic activity of environmental air samples from different rural, urban and industrial sites in Flanders, Belgium

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Abstract

The present study reports mutagenic and genotoxic activities associated with ambient air collected at 15 sites characteristic for urban, industrial or rural conditions in Flanders. Airborne particulates (PM10) and semi-volatile compounds were collected on quartz filters (QF) and polyurethane foam (PUF) cartridges using a high-volume sampling device. The mutagenic and genotoxic potency of the organic extracts – Soxhlet extraction with acetone – was determined by use of the *Salmonella* mutagenicity standard plate-incorporation assay and the Vitotox® assay, respectively. Concentrations of 16 polycyclic aromatic hydrocarbons (PAHs) in the extracts were determined by reversed-phase high-performance liquid chromatography (HPLC).

Ambient air samples contained significant PAH levels and mutagenic activities at all 15 sites: direct mutagenicity of up to 47 revertants per cubic meter was found in the QF extracts and more limited activity of up to 11 rev m^{-3} in the PUF extracts. Metabolic activation of PUF extracts resulted in an important increase in mutagenic activity, up to 30 rev m^{-3}, but no such increase was observed for QF extracts. The highest values were observed outside large cities at industrial sites and at a rural site contaminated by pollution from a chemical plant at a distance of 4 km. Also at the background location near the North Sea a significant mutagenic activity was measured in the QF extracts (+S9: 9 rev m^{-3}; −S9: 7 rev m^{-3}). Apparently, there is in Flanders a significant background exposure level to airborne mutagenicity, even in areas with limited or no nearby pollution sources. Based on the concentrations of 10 mutagenic PAHs and supposing additivity of their specific mutagenicities, only a few percent (mean 3%) of the observed indirect mutagenic activity could be explained. This implies that most mutagenic activity originated from other substances that were not identified or measured in our chemical analysis. This underscores the importance of bio-monitoring measurements.

Keywords: Airborne particles (PM10); Industrial point-sources; *Salmonella* mutagenicity assay; Genotoxicity; Polycyclic aromatic hydrocarbons; Vitotox® assay

1. Introduction

Despite efforts to improve air quality and to reduce atmospheric emissions in industrialised countries, air pollution is still considered to be an important cause of adverse health effects [1,2]. Although emissions from industry, power plants and incinerators has decreased

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during the last decade, the increasing consumption of fossil fuels and the intensifying traffic suggest that carcinogens continue to be released into the atmosphere in significant quantities. Different studies tend to suggest that the incidence of lung cancer is elevated in urban and industrial areas [3–5]. An excess risk for lung cancer and cardiopulmonary diseases is associated with airborne carcinogens and coarse (PM10-2.5) or fine (PM2.5) particulate matter [6–8]. Indeed, PM10 particles have the ability to penetrate and deposit in the tracheo-bronchial and alveolar regions of the respiratory tract and carcinogens such as polycyclic aromatic hydrocarbons (PAHs) are adsorbed on their surface. Many of these PAHs and their derivatives are carcinogens and mutagens, and several of these compounds are classified as probable (2A) or possible (2B) human carcinogens by the International Agency for Research on Cancer (IARC) [9].

Residents of industrialised and densely populated regions such as Flanders (Belgium) are exposed daily to ambient air pollution. Many carcinogenic compounds are directly released into the environment by combustion processes, others are created from primary emitted compounds by chemical and photochemical reactions in the atmosphere [10,11]. Identifying and estimating human exposure to these mutagenic and genotoxic compounds is imperative to assess public health risk. However, due to the complexity of the mixture of pollutants and their potential interactive effects these risks cannot be adequately assessed using chemical analysis alone. It is suggested that in addition to conventional chemical monitoring, biological effect measurements (e.g. mutagenicity assays) could considerably improve these risk assessments. These types of assay allow an integrated assessment of mutagenic or other effects of air pollutants, including effects of unidentified substances and possible interactions between the individual pollutants.

In a previous study [12], we reported on the mutagenic and genotoxic activities of ambient air samples collected at three sites in Flanders. To evaluate the levels of airborne mutagenicity/genotoxicity in Flanders, especially in relation to daily exposure of the population, samples from 15 other sites were evaluated in the present study. Seven were located in residential areas and are representative of general living conditions in Flanders. Eight other sites were industrial sites characterised by a specific point-source and with a minimal input from other nearby industrial or domestic emission sources. Airborne particles (PM10) and semi-volatile organic compounds were collected and analysed for PAHs. Concurrently, the mutagenic and genotoxic activities of these samples were evaluated using two bioassays: the *Salmonella* mutagenicity test/plate incorporation assay and the Vitotox® test. Potential relationships between observed biological effects and PAH concentrations were explored.

2. Experimental: materials and methods

2.1. Sites

Ambient air was sampled at 15 sites in Flanders—a densely populated (6 million inhabitants, 450 km^{-2}) and industrialised region of Belgium (Table 1). During the winter of 2002, seven (sites 1–7) locations were sampled representing residential neighbourhoods in industrial, urban or rural areas in Flanders. During the summer and autumn of 2002, eight strictly industrial locations (sites 8–15) were sampled, each of these characterised by a specific industrial point-source. Samples were taken downwind from the source.

The so-called 'residential' locations (1–7) were characterised by emissions arising mainly from traffic and residential heating, however, at several of these locations some industrial sources were identified. In 'Menen' (1): a small town, the sampling site was located near the southwest border of the city centre; at 800 m to the northeast of an incinerator and at the north (approximately 400 m) of a scrap yard (shredder). In 'Gent' (2): the second largest city in Flanders (230,000 inhabitants), the sampling site was situated in a small park close to the historical centre. In 'Zelzate' (3): a small town near the Ghent-Terneuzen Canal industrial zone, the sampling site was located at 550 m (NNE) of a petrochemical factory (heavy-fuel refinery) and at approximately 1000 m northwest from the city centre. In 'Vilvoorde' (4): a town at the northern border of the urban agglomeration of Brussels (1 million inhabitants), the sampling site was located in a cemetery near the city centre. In 'Nieuwpoort' (5): a tourist resort on the Belgian coast, the sampling site was located outside town in a marina with no habitation, traffic or other pollution sources between the sampling site and the sea. To represent background conditions, sampling at this station was always performed with winds blowing from the sea. In 'Olen' (6): a rural village, the sampling site was located just outside the village centre and at 1.2 km (NE) from an important industrial site (non-ferrous metal industry). In 'Leut' (7): a rural village, the sampling site was located in the centre at 4 km (NW) from a large chemical plant in Geleen (The Netherlands). Samples at all these stations were mostly taken with south-westerly winds except for the sample taken at the coastal site (5), i.e. NNE.

In the summer and autumn of 2002, eight other sites were sampled, each characterised by a specific industrial point-source. All sites were located in industrial zones at some distance from residential areas. The 'Antwerpen' (8) site was located in the port of Antwerp next to two large refinery plants. 'Gent2' (9) was situated next to a motorway, at 500 m northeast of an incinerator. 'Laakdal' (10) was located next to chemical industry and a motorway. The 'Kallo' (11) site was also situated in the port of Antwerp, 1 km south of a chemical plant. The 'Brugge' (12) site was close to an asphalt plant and the 'Wachtebeke' (13) site was located at 1 km of major

Table 1
Sampling conditions and the amount of particle matter (PM10) collected on filters[a]

Sample	Location	Type	Date	Wind speed (m/s)	Wind direction	Ambient temperature (°C)	Rain[b] (mm)	PM10[c] ($\mu g\,m^{-3}$)
1	Menen	Industrial	16-01-02	2.2	SSW	5.4	3.0	57.9
2	Gent	Urban	21-01-02	5.8	SSW	8.4	5.6	25.4
3	Zelzate	Industrial	28-01-02	8.1	SW-SSW	9.7	0.0	34.5
4	Vilvoorde	Urban	04-02-02	7.3	SSW	10.1	14.0	17.8
5	Nieuwpoort	Rural	13-02-02	5.6	NNE	4.1	0.0	20.0
6	Olen	Rural	20-02-02	6.2	SW-SSW	5.9	13.8	17.9
7	Leut	Rural	04-03-02	2.7	SE-WSW	7.8	0.0	49.2
8	Antwerpen	Point-source	04-09-02	2.0	WSW	18.1	0.0	59.7
9	Gent2	Point-source	07-09-02	3.9	SSW-S	18.0	14	18.6
10	Laakdal	Point-source	11-09-02	3.1	NE-ENE	16.1	0.1	28.1
11	Kallo	Point-source	14-09-02	3.1	N-NNE	16.6	0.0	25.7
12	Brugge	Point-source	30-09-02	2.5	SE	na	0.0	53.8
13	Wachtebeke	Point-source	19-10-02	4.2	WSW-SE	7.3	2.4	27.0
14	Kluisbergen	Point-source	28-11-02	3.4	S-SSW	9.0	3.4	33.1
15	Bree	Point-source	03-12-02	2.1	S	5.4	4.6	48.1

[a] na: not available.
[b] Amount of rain during 48 h.
[c] Total amount of PM10 collected in 48 h (two QF filters).

steelworks. 'Kluisbergen' (14) was located at 700 m from a coal-fired power-station and the 'Bree' (15) site was located at a metalwork factory. All industrial samples were taken downwind from the main source.

As the study was designed to cover as many different types of locations geographically spread over Flanders, each location was sampled only once for 48 h. Table 1 provides an overview of the sites and the conditions during sampling.

2.2. Sampling and sample preparation

The samples were taken using a high-volume air sampler (Digitel) with a size-selective inlet (PM10). The sampler inlet was located at street level (1.8 m height). Particulate matter and semi-volatile compounds were collected on quartz filters (QF20 Schleicher & Schuell) and polyurethane foam (PUF) cartridges, placed in series. The more volatile compounds, which were not trapped on the filter were retained in the PUF cartridges. The latter were pre-cleaned by 24-h Soxhlet extraction with acetone (CAS 67-61-1). The samples were taken at a flow rate of 30 m^3/h (500 l/min) during 48 h, resulting in a total sampled air volume of about 1300 m^3 (normalised to standard conditions, i.e. 1013 mbar and 0 °C). All samples were taken during weekdays starting at about 9 a.m. The quartz filters were changed automatically after 24 h. The same PUF cartridge was used during the entire 48-h sampling period. The weight of the collected particulate matter on the filters was determined by weighing the filters before and after sampling (prior to weighing, 24 h conditioning at 20 °C in desiccators). After sampling filters were stored at −18 °C until extraction.

QF and PUF were extracted separately for 24 h using a Soxhlet apparatus containing 100 and 2000 ml of acetone (CAS 67-61-1), respectively. QF and PUF blanks were extracted in an identical manner. Extracts were concentrated to 5.0 ml under a gentle N_2 stream and by use of a turbovap concentrator. A 0.5-ml aliquot was taken for HPLC (high-pressure liquid chromatography) analyses, the remaining volume was evaporated and the residue dissolved in 4.5 ml dimethyl sulfoxide (DMSO) (CAS 67-68-5) and used in the bioassays. The extracts were stored at −80 °C prior to the bioassay.

2.3. PAH analyses

The organic extracts of the QF and PUF cartridges were analysed for the presence of 16 PAHs using HPLC [13]. The HPLC system consisted of a liquid chromatograph system (Waters, Milford, MA, USA), a fluorescence detector (Perkin-Elmer LC240) and a UV detector in series. Separation of the PAHs was accomplished by use of a Vydac 201TP column (250 mm × 4.6 mm). The detected and quantified PAHs (two- to five-ring) were: naphthalene (N), acenaphtylene (ACY), acenaphtene (ACE), fluorene (FL), phenantrene (PH), anthracene (AN), fluoranthene (F), pyrene (P), benz(*a*)anthracene (B[*a*]A), chrysene (CH), benzo(*b*)fluoranthene (B[*b*]F), benzo(*k*)fluoranthene (B[*k*]F), benzo(*a*)pyrene (B[*a*]P), dibenz(*a,h*)anthracene (DB[*ah*]A), benzo(*ghi*)perylene (B[*ghi*]PY) and indeno(1,2,3-*cd*)pyrene (I[*cd*]P).

2.4. Bioassays

The organic extracts of the QF and the PUF cartridges, dissolved in DMSO, were tested for their mutagenic and genotoxic activity in two different bioassays.

The *Salmonella* mutagenicity assay [14] was performed on both the QF and PUF extracts using the standard plate-

incorporation test [15,16] with *Salmonella typhimurium* strain TA 98, with and without exogenous metabolic activation (S9). Dimethyl sulphoxide was used as solvent control, whereas 4-nitroquinoline-*N*-oxide (0.7 μg/plate) (CAS 56-57-5) and benzo(*a*)pyrene (5 μg/plate) (CAS 50-32-8) were used as positive controls without (−S9) and with (+S9) exogenous metabolic activation, respectively. For the exogenous metabolic activation a 10% (v/v) S9 mix was prepared with Aroclor 1254-induced rat liver S9 homogenate (ICN) [16]. Each sample was assayed at three concentrations (due to limited test material) using three replicate plates per concentration. With a total volume of 1300 m^3 air sampled, the three test doses (100 μl/plate) 100, 50 and 25% (v/v) of the organic extract, corresponded, respectively, to 26, 13 and 6.5 m^3 of sampled air. Revertants were counted with Quantity One quantification software in combination with Gel Doc2000 equipment (Bio-Rad) after 48 h incubation at 37 °C. Scored revertants were presented as revertants per cubic meter of air sampled, calculated from the dose–response curves. Samples giving at least a two-fold increase in mutants at the highest concentration tested compared with the average yield of spontaneous revertants (for TA98, the number of spontaneous revertants was between 20 and 50) and showing a concentration-related response, were regarded as positive [16].

The Vitotox® test is a bacterial assay, similar to the SOS chromotest [17], based on the expression of repair genes induced by genotoxic agents. This bacterial SOS system is a DNA repair mechanism that responds directly and immediately to DNA damage. The bacterial strain used for this assay is the TA104 RecN2-4 strain [18] that has a *lux* operon of the luminescent marine micro-organism *Vibrio fischeri* under transcriptional control of the recN gene, which is part of the SOS repair system. Under the influence of a genotoxic compound, the RecN promotor is de-repressed, which results in expression of the *lux* operon and light production. Some chemicals directly interfere with the light-emission system and stimulate light emission without any genotoxic activity (false positives). Other chemicals are bacteriotoxic and decrease light intensity because the micro-organisms are killed. In this case, genotoxic effects might be masked (false negatives). To estimate these effects, the test substance is simultaneously tested on a constitutive bacterial strain, TA 104 pr1 [19]. This construct provides the organisms with a background light emission. Substances that interfere directly with light emission will be detected with this strain and the genotoxic response can be corrected for this direct interference. The Vitotox® test is performed in multi-well plates containing the bacterial mix and dilutions of the test substance. Assays were performed in the presence and absence of a microsomal enzymatic mixture S9 (see above). Luminescence is measured with an automated spectrophotometer. Results are analysed with a standard software package (Microsoft-Excel), which for each test concentration calculates:

(1) the maximal signal-to-noise ratio (S/N) for the Rec strain, with S/N being the light production of exposed cells divided by the light production of the untreated cells;
(2) the maxima signal-to-noise ratio for the pr1 strain;
(3) the ratios of (1) and (2), which is used to correct for toxicity and for direct induction of the *lux* genes (max S/N(recN2-4)/max S/N(pr1)).

Genotoxicity is assumed to be present when the results of the calculations (1) and (3) are equal or larger than 1.5, and when there is a good concentration–effect relationship [19].

2.5. Statistics and correlation analyses

For data analysis of the environmental samples, the non-parametric Wilcoxon matched pairs test was used for testing paired data, correlations were tested using the non-parametric Spearman test [20]. The mutagenic and genotoxic responses were related to the analyses of the 16 PAHs using partial least-squares projections to latent structures (PLS) models Simca-P 9.0 software, (Umetrics, Umeå, Sweden) [21]. PLS models allow an investigation of correlations between numerous, often correlated, input and process variables (X) and several result variables (Y). Relations between X and Y variables are found by simultaneous projections of both the X and Y spaces to a plane or hyperplane. PLS analysis results in model coefficients for the variables, called 'weights'. The weights for the X-variables indicate the importance of these variables in the modelling of Y. The R^2 of the model is a measure of the variance explained by the model, while Q^2 is a measure of the variance of the variables that can be predicted by the model. The resulting model can be used to predict the mutagenic or genotoxic responses based on measured chemical concentrations [22,23]. A first model was developed with all 16 input variables (PAHs). Next, the known indirect-acting mutagens, i.e. 10 of the 16 PAHs analysed, were related with the indirect-acting activity (+S9). Resulting models were evaluated with respect to their correlation coefficients (R^2) and predictive capacity (Q^2).

3. Results

3.1. Particle concentrations and meteorological conditions

Table 1 provides an overview of the meteorological conditions during sampling and summarises the quantities of airborne particles (PM10) collected on the filters. The amount ranged from 17.8 (sample 4) to 59.7 μg m^{-3} (sample 8) with an average of 34.4 ± 15.2 μg m^{-3}. The samples (4, 6 and 9) with the lowest PM10 concentrations were collected during days with heavy rainfall (>10 mm). The mean (48 h) ambient temperature varied from 4.1 to 18.1 °C, the average temperature during winter sampling (n = 10) was 6.9 and 17.2 °C during summer/autumn sampling (n = 5). The registered wind speeds ranged between 2.1 and 8.1 m s^{-1}, the average speeds during winter and summer sampling were, respectively, 4.8 and 2.9 m s^{-1}.

Table 2
Concentration (ng m^{-3}) of the PAHs in the organic extracts (QF + PUF)[a]

PAHs	Residential[b] Mean ± S.D.	Point-sources[c] Mean ± S.D.	Background[d]
Napthalene	1.76 ± 1.93	8.00 ± 6.13	0.44
Acenapthylene	0.55 ± 0.84	0.51 ± 0.82	0.30
Acenaphtene	0.14 ± 0.12	0.04 ± 0.08	0.04
Fluorene	4.27 ± 4.19	6.59 ± 8.08	3.89
Phenantrene	20.7 ± 17.8	73.1 ± 137	15.8
Anthracene	1.68 ± 1.90	1.16 ± 1.26	0.40
Fluoranthene	7.65 ± 9.01	8.48 ± 14.4	5.67
Pyrene	2.80 ± 1.32	3.51 ± 3.80	1.69
Benz(*a*)anthracene	0.54 ± 0.56	0.29 ± 0.13	0.02
Chrysene	1.35 ± 1.18	0.40 ± 0.16	0.24
Benzo(*b*)fluoranthene	0.96 ± 0.56	0.41 ± 0.28	0.08
Benzo(*k*)fluoranthene	0.54 ± 0.42	0.38 ± 0.38	0.07
Benzo(*a*)pyrene	0.62 ± 0.53	0.19 ± 0.18	0.09
Dibenz(*a,h*)anthracene	0.61 ± 0.64	0.28 ± 0.38	0.24
Benzo(*ghi*)perylene	1.15 ± 2.69	0.91 ± 0.91	0.21
Indeno(1,2,3-*cd*)pyrene	2.69 ± 2.28	0.57 ± 0.36	0.46

[a] Sample types: QF, quartz filter; PUF, polyurethane foam.
[b] Mean ± standard deviation of concentrations measured at the six residential locations.
[c] Mean ± standard deviation of concentrations measured at the eight point-source locations.
[d] Concentration measured at Nieuwpoort.

3.2. Chemical analyses

The concentrations of the 16 PAHs in the organic extracts (QF + PUF) are summarised in Table 2; presented are the average concentrations taken over the residential ($n = 6$) and the point-source ($n = 8$) locations, and the concentrations at the background location (5) (not comprised in the above-mentioned average values). More detailed data (individual QF and PUF samples) are available on: http://allserv.ugent.be/~vdufour. Analysis of the PAH profiles showed that concentrations of the low-molecular-weight PAHs (up to pyrene, mw 202.26) were 5–10 times higher than the high-molecular-weight PAHs (from B[*a*]A, mw 228). FL, PH, F and P were the most abundant low-molecular-weight PAHs, and CH, B[*b*]F and I[*cd*]P the most abundant high-molecular-weight PAHs.

The PAH concentrations and profiles at the individual locations are not discussed in detail here, but the most relevant results for the present study are highlighted. The lowest levels were observed at 'Olen' (6), 'Vilvoorde' (4) and 'Nieuwpoort' (5), respectively, an industrial area, an urban site and the background location. For these locations the individual PAH concentrations did not exceed 0.5 ng m^{-3} except for the most abundant substances (FL, PH, F and P). The highest concentrations were measured at the industrial locations 'Menen' (1) and 'Zelzate' (3), and also at the rural/industrial location 'Leut' (7). In particular, the high-molecular-weight PAHs (≥benz[*a*]anthracene, mw 228) were 3–10 times more abundant compared with the other locations.

3.3. Mutagenic activity—Salmonella test

Results of the mutagenicity assay of the QF and PUF extracts are shown in Table 3. Mutagenic responses are expressed as specific mutagenicity, i.e. number of revertants per m^3 (±95% confidence limits) obtained from the dose–response curves through regression analysis [20]. Due to the limited number of concentrations tested the resulting regression analyses (slope) may lack precision. The raw data (mean number of revertants per dose ± standard deviation) are available on: http://allserv.ugent.be/~vdufour. The positive controls, 4-nitro-quinoline-*N*-oxide (0.7 μg/plate) and benzo(*a*)pyrene (5 μg/plate) induced an average response of 780 ± 91 and 502 ± 35 rev/plate, respectively.

The direct (−S9) and the indirect (+S9) mutagenic potency differed with sample type (QF or PUF) and location. Without metabolic activation all filter extracts showed direct mutagencity activities ranging from 4.9 to 46.8 rev m^{-3} with an average of 17.2 rev m^{-3}. With metabolic activation, the mutagenic responses were similar; ranging from 3.1 to 42.7 rev m^{-3} with an average of 17.0 rev m^{-3}. In the PUF extracts, the activities observed were significantly ($P < 0.001$) lower and most PUF extracts showed only mutagenic activity

Table 3
Results of the *Salmonella* test: mutagenicity (response ± 95% confidence limits) of QF and PUF samples[a] with (+S9) and without (−S9) metabolic activation, and the calculated activity[b]

Sample	QF (rev m^{-3})		PUF (rev m^{-3})[c]		Calculated activity[d] (rev m^{-3})
	−S9	+S9	−S9	+S9	+S9
1	27.8 ± 2.0	37.0 ± 2.9	–	–	1.4 (3.6)
2	21.4 ± 1.4	17.6 ± 1.3	–	–	0.4 (2.4)
3	16.8 ± 1.2	15.5 ± 1.9	4.7 ± 0.8	14.6 ± 1.4	1.5 (5.6)
4	7.6 ± 1.0	5.8 ± 1.6	–	–	0.3 (4.2)
5	8.9 ± 0.6	7.0 ± 0.9	–	–	0.2 (2.5)
6	6.6 ± 0.8	6.6 ± 1.2	–	–	0.3 (3.0)
7	46.8 ± 3.9	42.7 ± 1.8	10.9 ± 0.9	29.7 ± 3.8	1.2 (1.7)
8	9.9 ± 0.5	10.8 ± 2.0	5.3 ± 0.5	–	1.0 (7.8)
9	9.9 ± 0.6	3.1 ± 0.1	2.87 ± 0.24	4.6 ± 0.4	0.2 (3.1)
10	13.8 ± 1.4	13.5 ± 2.3	4.5 ± 0.6	5.6 ± 0.3	0.2 (0.9)
11	4.9 ± 0.4	3.6 ± 0.6	–	2.9 ± 0.3	0.1 (1.3)
12	25.1 ± 1.3	22.1 ± 3.4	–	6.5 ± 0.7	0.4 (1.5)
13	35.3 ± 2.1	22.2 ± 1.9	–	5.0 ± 0.5	0.5 (1.9)
14	11.0 ± 1.2	22.4 ± 1.8	–	5.9 ± 0.4	0.3 (2.9)
15	12.5 ± 0.5	24.9 ± 2.0	–	4.5 ± 0.5	0.5 (2.1)

[a] Sample type: QF, quartz filter; PUF, polyurethane foam.
[b] The activity calculated from the specific activity of 10 known mutagenic PAHs and their concentrations measured in the QF + PUF extracts.
[c] (–): no mutagenic response.
[d] The values between parentheses specify the % of the observed total (QF + PUF) mutagenic activity (+S9) explained by the calculated activity.

with metabolic activation. Nevertheless, the few ($n = 5$) PUF samples exhibiting direct-acting activity had an average response of 5.6 ± 3.1 rev m^{-3}. Addition of S9 significantly ($P < 0.05$) increased mutagenic responses, nine positive PUF samples induced an indirect response, the corresponding activities ranging from 2.9 to 29.7 rev m^{-3}, with an average of 8.8 rev m^{-3}.

The resulting total (QF + PUF) mutagenic activities ranged from 4.9 up to 72.8 rev m^{-3}. The highest total response was found at the rural/industrial location 'Leut' (7) both with the direct and indirect activity measurements. Other sites indicating high direct and indirect activities were the industrial locations 'Menen' (1) and 'Zelzate' (3), and the point-source locations 'Brugge' (12) and 'Wachtebeke' (13). Elevated activities were also observed at the industrial sites 'Kluisbergen' (14) and 'Bree' (15), but here activities were mainly indirect. Considering only winter samples, the mutagenicity levels measured at the background location 'Nieuwpoort' (5) were the lowest (<10 rev m^{-3}) observed in this study, however, comparable activities were also observed at the urban location 'Vilvoorde' (4) and the industrial location 'Olen' (6). The lowest overall activity was measured at the point-source location 'Kallo' (11), in a summer sample.

In addition to the results obtained with the *Salmonella* assay, the 'calculated activity' is presented in Table 3. The calculated activity represents the indirect mutagenicity caused by the ten measured known mutagenic PAHs (F, P, B[*a*]A, CH, B[*b*]F, B[*k*]F, B[*a*]P, DB[*ah*]A, B[*ghi*]P, I[*cd*]P) and is obtained by multiplying their specific activities (revertants per microgram) reported in the literature [24–27] with the corresponding concentrations in QF + PUF extracts, as detailed on http://allserv.ugent.be/∼vdufour. The calculated activities ranged from 0.1 up to 1.5 rev m^{-3}, representing between 0.9 and 7.8% (Table 3: values between parentheses) of the measured mutagenic activity (average, 3.1%). These calculated activities are only valid under the assumption of additivity, i.e. the mutagenicity of the mixture is simply assumed to be the sum of the expected effects from each individual compound, i.e. no synergistic or antagonistic interactions are taken into account. The literature describing the behaviour of genotoxic compounds in mixtures is sparse and, occasionally, contradictory. White [28] concluded on the basis of his own experimental results and literature data that mutagenic risks posed by simple, well-characterised mixtures of PAHs can reasonably be estimated as the sum of the risk of the individual PAHs. Other authors also demonstrated that combined mutagenic effects (*Salmonella* mutagenicity) in PAH mixtures [29] and in diesel exhaust particles (DEPs) spiked with PAHs [30,31], are additive. In contrast, in other studies rather synergistic [32] and antagonistic [33] effects were observed.

Table 4
Results of the Vitotox® assay: genotoxicity of QF and PUF samples with (+S9) and without (−S9) metabolic activation[a]

Sample	QF[b]					PUF[b]				
	LOEC (%)		rec/pr1 per m^3		EC_{50} (%)	LOEC (%)		rec/pr1 per m^3		EC_{50} (%)
	−S9	+S9	−S9	+S9		−S9	+S9	−S9	+S9	
1	0.25	–	31.90	–	0.20	X	–	X	–	0.07
2	0.5	–	13.70	–	0.58	X	–	X	–	0.09
3	0.5	–	13.70	–	0.53	X	–	X	–	0.07
4	0.5	–	11.93	–	1.10	–	–	–	–	0.58
5	1	–	7.23	–	0.66	X	–	X	–	0.07
6	0.5	–	12.77	–	0.54	X	–	X	–	0.08
7	1	–	6.13	–	0.37	1	–	7.00	–	0.36
8	0.5	–	12.52	–	0.71	X	–	X	–	0.11
9	0.25	–	22.69	–	0.35	X	–	X	–	0.25
10	0.5	–	13.05	–	0.24	X	–	X	–	0.16
11	–	–	–	–	1.40	X	–	X	–	0.35
12	0.25	1	47.2	6.15	0.16	–	–	–	–	1.10
13	0.5	1	16.27	5.54	0.24	X	–	X	–	0.32
14	0.5	–	15.14	–	0.45	X	–	X	–	0.46
15	0.5	–	17.4	–	0.47	–	–	–	–	0.99

[a] LOEC: lowest observed effect concentration; EC_{50}: concentration where cell activity has dropped to 50%,(S/R = 0.5); (–): no genotoxic effect; (×): toxic, no sufficient bacterial growth.
[b] Sample type: QF, quartz filter; PUF, polyurethane foam.

3.4. Genotoxic activity assessed by the Vitotox® assay

With the Vitotox® assay the samples were tested at four concentrations, 0.13, 0.25, 0.50 and 1% (v/v) of the organic extracts corresponding to, respectively, 0.034, 0.065, 0.13 and 0.26 m^3 of air sampled. The evaluation for genotoxicity (LOEC and rec/pr1 signal per m^3) and toxicity (EC_{50}) is given in Table 4. Based on the calculations, as explained in Section 2, the genotoxic effects are presented as the lowest test dose giving a genotoxic result, lowest observed effect concentration (LOEC) and the response (signal to noise ratio of the *Rec* and *Pr*1 strain at the observed LOEC) per cubic meter air.

In the Vitotox® assay without metabolic activation most PUF extracts were toxic, resulting in an impaired bacterial growth insufficient to show any significant genotoxic effect. While addition of S9 resulted in detoxification of these samples, no indirect genotoxic activity was detected. In contrast to the PUF extracts, the QF extracts did show genotoxic responses. Only at the highest test concentration some QF samples showed toxic effects, possibly reducing the genotoxic responses. Metabolic activation (+S9) significantly reduced the genotoxic responses in QF samples: only two industrial samples (12 and 13) were still genotoxic after addition of S9, and only for the highest test concentration.

3.5. Modelling of mutagenicity and genotoxicity

Correlation analysis using PLS modelling was used to relate the mutagenic and genotoxic activity to the chemical composition of the samples (Table 5). Models based on the PAH concentrations and the *Salmonella* mutagenicity test results of the filter samples predicted the indirect mutagenicity ($Q^2 = 0.827$) better than the direct activity ($Q^2 = 0.689$). The observed versus predicted indirect mutagenic activity (rev m^{-3}) obtained with this model ($Q^2 = 0.827$) is shown in Fig. 1. Selecting only the data of the 10 known indirect-acting mutagenic PAHs did not improve the prediction of the indirect activity ($Q^2 = 0.799$). The models correlating the mutagenicity data and chemical analyses of the PUF samples

Table 5
Summary of the PLS models developed, relating PAH concentrations and mutagenic responses

Result variable *Y*	No. of *X* variables	R^2	Q^2
+S9, QF	16	0.952	0.827
+S9, QF	10	0.903	0.799
−S9, QF	16	0.912	0.689
+S9, QF + PUF	16	0.912	0.634
+S9, QF + PUF	10	0.912	0.688
−S9, QF + PUF	16	0.849	0.439

Y: mutagenic/genotoxic responses; *X*: PAH concentrations; R^2: correlation; Q^2 variance.

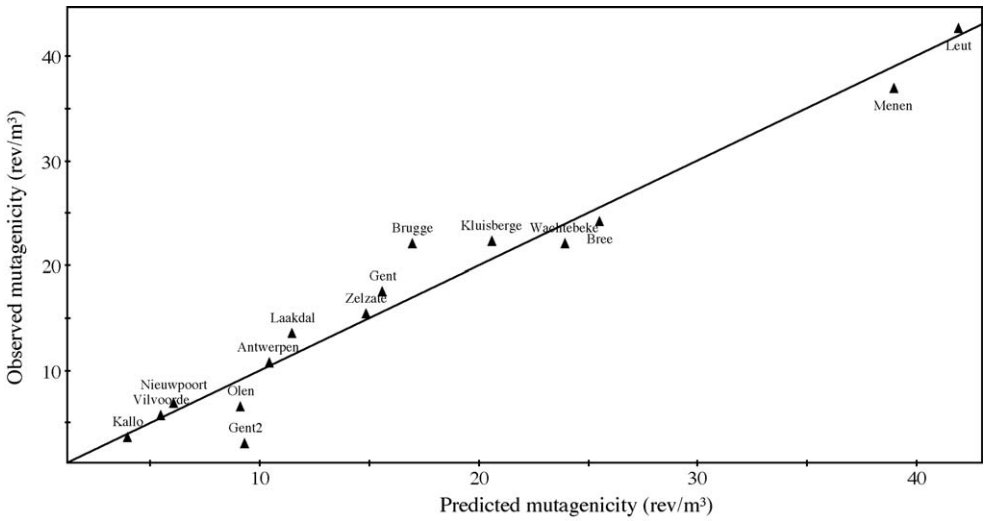

Fig. 1. The predictive capacity of the PLS model ($Q^2 = 0.827$) indicating observed vs. the predicted indirect mutagenicity.

predicted direct activity ($Q^2 = 0.854$) quite well, but not the indirect activity ($Q^2 = 0.438$). The correlations or predictions did not improve by relating the indirect mutagenicity with the 10 mutagenic PAHs only.

The analysis based on the data of the PUF samples did not result in statistically significant models, probably due the limited biological activities measured in the PUF extracts.

A good model was obtained for the total (QF + PUF) indirect-acting mutagenic activity based on the corresponding total concentrations of the 16 measured PAHs ($Q^2 = 0.634$). The correlation and predictive capacity were similar when the model was based on the 10 known mutagenic PAHs. A similar model for the total direct-acting activity was less powerful ($Q^2 = 0.439$).

Due to the limited genotoxicity data obtained with the Vitotox® test, PLS analysis was restricted to the results of the QF samples without metabolic activation. However, no significant model could be obtained with these data.

4. Discussion

The *Salmonella* mutagenicity assay has been used frequently to measure airborne mutagenicity, and provides reproducible results that correlate with measured PAH concentrations [34,35]. The Vitotox® assay is a new bacterial genotoxicity test that was previously shown to be a very rapid and sensitive screening assay for pure chemicals [19]. However, applications with complex environmental mixtures are few in number [12,36]. The results obtained with the Vitotox® test demonstrated toxic effects for the PUF samples without metabolic activation, masking possible genotoxic responses. Because of these toxic effects and the fact that metabolic activation seems to reduce genotoxic activity (see Section 3.4), the use of the Vitotox data in this study was limited to the QF samples without metabolic activation (−S9).

The complex environmental mixtures to which we are exposed through air pollution are difficult to identify and quantify. The chemical analyses limited to 16 PAHs, provide us with specific information about a number of known mutagenic and carcinogenic PAHs [9,23–27] and give an idea about the general levels of organic pollution. The low-molecular-weight PAHs were the most abundant, the higher molecular-weight PAHs such as B[*a*]P were only present at minor concentrations. But because of their significant mutagenic/carcinogenic potency, the high-molecular-weight PAHs are, however, of greater concern [9]. In many studies, B[*a*]P has been used as indicator compound for PAH exposure due to its strong carcinogenicity and close correlation with other PAHs [37,38]. With an average B[*a*]P concentration of $0.62 \pm 0.53\,\mathrm{ng\,m^{-3}}$ for the residential locations ($n = 6$) and $0.19 \pm 0.53\,\mathrm{ng\,m^{-3}}$ for the point-source locations ($n = 8$), B[*a*]P levels observed in the present study were among the lowest reported in Europe. Levels reported in Birmingham (UK) were $0.81 \pm 0.65\,\mathrm{ng\,m^{-3}}$ in winter and $0.25 \pm 0.20\,\mathrm{ng\,m^{-3}}$ in summer [39] and in Heraklion (Greece) the B[*a*]P concentrations ranged between 0.14 and 3.47 with an average of $1.16\,\mathrm{ng\,m^{-3}}$ during the period 2000–2002 [40].

To assess geographic differences in ambient air quality in Flanders, different types of site were monitored. Although the sampling campaign was probably too limited (one 48-h sample per location) to characterise

mutagenic exposures in detail or to discuss seasonal and regional variations, the results obtained provide new and sometimes remarkable information about ambient mutagenicity levels in Flanders. Samples taken at the industrial and urban sites were expected to contain more high-potency mutagenic compounds than those from the rural sites. Surprisingly, the highest direct and indirect mutagenic activity was found in a sample taken at the rural/industrial location 'Leut' (7), respectively, 57.7 and 72.4 rev m^{-3}. The elevated mutagenic potency of this sample was also associated with a genotoxic response in the Vitotox assay: of all PUF samples analysed only this one (as discussed above) induced a genotoxic response (−S9). This rural site is situated near the Dutch border at 4 km from the largest chemical plant in the Netherlands; during sampling the wind blew half the time from the direction of the chemical plant, which could explain the elevated biological activities. Other locations with important biological activities (see Table 3) were the industrial sites 'Menen' (1) and 'Zelzate' (3) and the point-source locations 'Brugge' (12) and 'Wachtebeke' (13). A recent study performed in Baden-Württemberg (Southern Germany) described comparable mutagenicity levels for eight urban and residential locations [41]. The highest reported activities were detected at a roadside location in the city of Karlsruhe where, over a period of 2 years, the average activity ($n = 21$) was 32.7 rev m^{-3} with a maximum of 101.5 rev m^{-3}. For other urban sites in Mannheim, Stuttgart and Reutlingen, which were not roadside locations but still had significant traffic emissions, mean activities of 19.6 rev m^{-3} (max 74.5), 17.8 rev m^{-3} (max 58.4) and 20.7 rev m^{-3} (max 85.5), respectively, were reported.

In general, the samples with the highest biological activity (1, 3, 7, 12 and 13) were characterised by higher PAH concentrations, i.e. mainly the high-molecular-weight and more biologically potent PAHs. The sampling sites 'Menen' (1) and 'Zelzate' (3) were both situated at the border of an urban-residential area but close to industrial sources zones harbouring an incinerator and a scrap plant (Menen) and a petrochemical plant (Zelzate). The mutagenic activity determined in Menen (1) was only found in the QF extract, in Zelzate (3) the activity was found in both the QF and PUF extracts. The PAH profile of Zelzate (3) was characterised by an elevated concentration of fluoranthene in the PUF extract, which contributed to its mutagenic activity. Fluoranthene is an indirect-acting mutagen with a specific activity of 0.0194 rev μg^{-1} in the *Salmonella* test [25–28]. Theoretically, this contributed approximately 3.4% of the observed indirect activity. Together with the other nine know mutagenic PAHs [25–28], 4.3% of the activity measured was explained. Although PAH concentrations in the point-source samples 'Brugge' (12) and 'Wachtebeke' (13) were somewhat lower, they showed relatively high direct- and indirect-acting mutagenicity. Moreover, in the Vitotox® assay these samples induced high genotoxic activities and were the only two producing significant indirect-acting (+S9) responses. Based on the PAH analyses, no obvious explanation could be provided; probably the genotoxic agents originating from the selected point-sources (asphalt central and a major steelworks) did not correlate with the measured PAH levels.

The site at the coast 'Nieuwpoort' (5) was selected to represent background conditions during winter as the sample was taken downwind from the sea. In the resulting PUF sample, no biological effects were detected but the QF sample did exhibit low but significant mutagenic and genotoxic activity. Since no nearby pollution sources were identified, the observed biological activities probably originated from distant sources (long-range trans-boundary pollution). The activities reported by Erdinger et al. [41] for their background location 'Kälbelescheuer' situated in the Black Forest (Southern Germany) were much lower, with activities ranging from 0.0 to 8.57 rev m^{-3} with a mean (2 years, $n = 21$) activity of 2 rev m^{-3}. At the urban site 'Vilvoorde' (4) and the rural/industrial site 'Olen' (6), i.e. with local pollution sources such as congested traffic, residential heating and a large non-ferrous metal industry, the activities measured were of the same magnitude as those at the background site (5). The weather conditions during sampling at these stations (see Table 1), i.e. a relatively high wind speed enhancing dilution and dispersion and an important level of precipitation, resulted in a level of biological activity similar to the background activities of 'Nieuwpoort'. Indeed, as described in Section 3.2, the PAH levels and profiles at these three sites (5, 4 and 6) were very similar during mild winter conditions (Table 1). This indicates pollution is widespread in Flanders and exposure may be quite homogenous. At the point-source location 'Kallo' (11), sampled during summer, mutagenic activity was similar to the activities found in 'Nieuwpoort' (5), 'Vilvoorde' (4) and 'Olen' (6). However, the indirect-acting activity of the 'Kallo' sample (11) was not only present in the QF extract but also in the PUF extract. The indirect-acting activity in the PUF sample could be explained by the presence of high-molecular-weight PAHs, i.e. 96% of the total B[*a*]A and 77% of the total CH content. This shift of high-molecular-weight PAHs from the particle- to the gas-phase has been associated with high-volume sampling in combination with elevated ambient temperatures [42,43]. In our previous campaign, similar effects

were described for samples collected during summer [12]. Loss of these high-molecular-weight PAHs from the collecting filter resulted in very low concentrations, probably also reflected in the absence of any genotoxic response in the QF extract of 'Kallo' (11).

As discussed, the levels of B[*a*]P measured at all stations were rather low. However, the mutagenic activity was much higher than expected on the basis of the benzo(*a*)pyrene concentrations only. The mutagenic activity (+S9) per nanograms B[*a*]P of air samples taken in a busy street in Copenhagen was on average 13.4 revertants per nanogram [44], and a similar number of 12.3 ± 21.7 revertants per nanogram B[*a*]P [45] has been reported more recently. The values in our study were much higher, with on average 77 revertants per nanogram B[*a*]P. That mutagenic activity in the present study did not reflect the decrease in B[*a*]P concentration might be the result of emissions containing relatively more other mutagenic chemicals, including substituted PAHs derived from diesel engines [46], and of differences in atmospheric conditions. Higher sunlight intensity with elevated ozone and nitrogen oxide concentrations enhance atmospheric transformations of PAHs, especially B[*a*]P [47,48].

There are indications that the mutagenicity of particles collected in ambient air is not always correlated with the PAH content or the PAH indicator B[*a*]P [49,50]. In this study the sum of the concentrations of the 10 indirect-acting PAHs correlated quite well with the indirect (+S9) total mutagenic activity ($r = 0.76$, $P < 0.001$). The indirect mutagenicity was also correlated ($r = 0.69$, $P < 0.005$) with the B[*a*]P concentration, but less so compared with the correlation ($r = 0.93$, $P < 0.001$) reported by Feilberg et al. [45]. The best correlation was found with B[*ghi*]P ($r = 0.85$, $P < 0.001$) which is a good indicator for traffic emissions, a dominant source of air pollution in our regions. The PLS analyses demonstrated also good correlations between the PAH concentrations and the observed mutagenic activities. It is, however, uncertain whether the resulting complex PLS models, comprising different coefficients for each PAH, could predict mutagenic activity in other settings. When assuming that PAH mutagenicity is additive, measured concentrations of 10 mutagenic PAHs could only explain a few percent (0.9–7.8%, with a mean of 3.0%) of the observed mutagenic activity (see Table 3). This implies that most mutagenic activity stemmed from other substances that were not identified nor measured in our chemical analysis. It is thus clear that measurement of mutagenic activity in conjugation with chemical analyses is required to fully assess the health impact of air pollutants. Indeed, new or different emissions or changes in atmospheric reactions may affect mutagenic activity associated with air pollution to a large extent, while chemical analyses might not reflect these changes adequately.

In conclusion, the mutagenic and genotoxic evaluation of ambient air in Flanders showed significant activities at all selected sites, including the (coastal) background site (5). Together with the high activities observed at the rural/industrial site 'Leut' (7) contaminated by an industrial plant at 4 km distance, this illustrated the importance of long-distance transport of airborne pollutants. High exposure to airborne mutagens in rural areas was also demonstrated during our first campaign. The high mutagenic activities in the rural municipality Peer [12], possibly indicating adverse consequences for public health, was consistent with the result of a bio-monitoring study performed in that same village [51]. The rural population showed higher levels for some exposure biomarkers and had also significantly higher hypoxanthine phosphoribosyltransferase (*HPRT*) gene mutant frequency than the population residing in an urban industrial area.

The presence of relatively high mutagenic activity associated with air pollutants in Flanders was in contrast with relatively low chemical readings. For many samples, the B[*a*]P concentrations were near detection limit although significant biological activities were still observed. The PAH content was in most cases an indication of potential biological activity, but not in all cases, emphasizing the value of bioassays in air-quality monitoring.

The presence of high-molecular-weight PAHs and of biological activity in the PUF extracts, as observed for several (summer) samples, demonstrated the importance of using a back-up adsorber. Air-monitoring studies based only on particle collection (filter) probably underestimate the actual exposure level, particularly during summer.

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